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2, 4- DIACETYLPHLOROGLUCINOL (DAPG) A SECONDARY METABOLITE OF FLUORESCENT PSEUDOMONADS AS A POTENTIAL BIOCONTROL AGENT FOR PLANT DISEASES

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ABSTRACT:

Biocontrol using antagonistic bacteria has been considered as an alternative strategy to agrochemicals that are harmful to human health and the environment. Secondary metabolites with biocontrol properties have been reported from diverse members of the beneficial rhizosphere flora; however, those produced by the fluorescent pseudomonads have received the most attention. ***Pseudomonas fluorescens*** produce a variety of inhibitory metabolites like indole-3- acetic acid, siderophores, phenazine-1-carboxylic acid (PCA), pyocyanin, 2-acetamidophenol, pyrrolnitrin, pyoluteorin, Phenazine-1-Carboxylic Acid, 2, 4-diacetylphloroglucinol (2, 4-DAPG), viscosinamide, etc. Among these 2, 4-DAPG acts as a good potential biocontrol agent for controlling plant diseases. It exhibits biocontrol activity against phytopathogens such as oomycetes (particularly *Pythium spp.*), fungi (*Fusarium oxysporum, Gaeumannomyces, graminis, Rhizoctonia solani*, *Thielabiopsis basicola* etc.), and to a lesser extent bacteria (e.g. *Pectobacterium carotovorum*) and nematodes (e.g. Meloidogyne spp.). This article focuses on antimicrobial metabolites 2, 4-diacetylphloroglucinol produced by fluorescent pseudomonads, discusses their role in suppressing diseases of important crops and reviews the prospects of physiochemical factors influencing production to improve the efficacy of these biocontrol agents.

KEYWORDS: Biocontrol, Phytopathogens, Rhizosphere, Fluorescent Pseudomonads, Physiochemical.

INTRODUCTION:

The rhizosphere is the narrow zone of soil surrounding plant roots and is directly influenced by root secretions and associated soil microorganisms. It promotes large active groups of bacteria known as plant growth promoting rhizobacteria (PGPR) [1]. These organisms are known to rapidly colonize the rhizosphere [2] and mediate improved plant growth by stimulation of the plant or through biological control of pathogens or induction of host defense mechanisms. Among these organisms, *Pseudomonas fluorescens* are considered to be the most potential group of plant growth promoting rhizobacteria involved in biocontrol of plant diseases [3].

The antibiotic 2, 4-diacetylphloroglucinol (2, 4-DAPG) produced by ***Pseudomonas fluorescens*** inhibit a broad spectrum of plant pathogenic fungi [4] and control a variety of root and seedling diseases. Various evidences validate the importance of 2, 4- DAPG production in biological control e.g. mutation in the biosynthetic pathway results in reduced biocontrol activity[5], population size of 2, 4-DAPG producers in the rhizosphere correlated with disease suppressive of the soil and in situ antibiotic production [6], diverse 2, 4-DAPG-producing *Pseudomonas* spp. have been isolated from the rhizosphere of various crop plants, and their roles in promoting plant growth and inhibiting root diseases are the subjects of current investigations worldwide.

PSEUDOMONAS FLUORESCENS AS BIOCONTROL AGENT*:*

Pseudomonas fluorescens is a motile, nonsporulating, rod shape, gram negative obligate aerobic bacteria that inhabits many environments, including: plants, soil, and water surfaces. The GC content in the DNA of strains belonging to *P. fluorescens* subgroups varied from 61.2 to 64.5%, and their DNA-DNA homology with the type strain *P. fluorescens* ATCC 13525 was 24 to 83% [7].

Pseudomonas fluorescens during root colonization produce a variety of inhibitory substances. Plant growth-promoting capability of these bacteria is largely because of the production of indole-3- acetic acid [8], siderophores [9] and antibiotics [10]. Production of antibiotics for example phenazine-1-carboxylic acid (PCA), pyocyanin, 2-acetamidophenol, pyrrolnitrin, pyoluteorin, Phenazine-1-Carboxylic Acid, 2, 4-diacetylphloroglucinol, viscosinamide and tensin in different species of Pseudomonad has been reported. Among these antimicrobial compounds, 2, 4-DAPG is a phenolic derivative with antifungal, antibacterial, antiviral, and phytotoxic properties that has been intensively studied. Besides its anti-microbial activity, 2, 4-DAPG induces systemic resistance in plants and promotes exudation of amino acids from roots [11]. They have been mostly studied for protection of crop

plants from phytopathogenic oomycetes (particularly *Pythium spp*.) and fungi (*Fusarium oxysporum, Gaeumannomyces, graminis, Rhizoctonia solani*, *Thielabiopsis basicola* etc.), and to a lesser extent bacteria (e.g. *Pectobacterium carotovorum*) and nematodes (e.g. Meloidogyne spp.). Disease suppression by these bacteria often entails inhibition of phytopathogens in soil or on roots, by competition or antagonism [12]. Plant protection may also result from direct interactions with the host plants, especially in the case of induced systemic resistance (ISR) [13].

SECONDARY METABOLITES OF *PSEUDOMONAS* INVOLVED IN THE BIOCONTROL OF PLANT DISEASE:

Metabolites implicated in biocontrol appear to be broad-ranging in their inhibitory action [14]. They produce secondary metabolites like as antibiotics [15], phytohormones, volatile compound Hydrogen cyanide (HCN), and siderophores [16]. Phloroglucinols and phenazines have been shown to inhibit wide range of fungal pathogens in the laboratory.

Siderophores are defined as relatively low molecular weight, ferric ion specific chelating agents elaborated by bacteria and fungi growing under low iron stress. Siderophores exhibit both fungistatic and bacteriostatic effects in the laboratory under conditions of low iron. Siderophores are produced by many microorganisms. The role of these compounds is to scavenge iron from the environment and to make the mineral, which is almost always essential, available to the microbial cell. Pseudomonads produce a range of iron-chelating compounds including salicylic acid, pyochelins and fluorescent pseudobactins and pyoverdines. Fluorescent siderophores are unique to pseudomonads - a trait that has implicated these organisms as PGPR. Fluorescent siderophores have been isolated from soil [17] and there is considerable genetic and biochemical evidence that demonstrates their role in the promotion of plant growth and in biocontrol [18].

Many rhizosphere strains also produce ammonia [19] and HCN that acts as important metabolites in biocontrol. Some species of *Pseudomonas* can produce levels of HCN *in vitro* that are lethal to certain pathogenic fungi, e.g. *Thielabiopsis basicola,* and, thus, prevent black root-rot of tobacco. High concentrations of HCN are toxic to some plants and it has been suggested that fluorescent pseudomonads producing HCN may be responsible for a reduction in the yield of certain crops, e.g. potato [20]. Some plant hormones such as salicylic acid, a precursor of pyochelin [21] and a siderophore [22], is concerned in the induction of systemic acquired resistance in plants [23]. Another plant hormone, indole acetic acid (IAA), is also produced by many strains that display biocontrol activities.

Disease-suppressive antibiotic compound characterized in ***Pseudomonas fluorescens*** include N-containing heterocycles such as phenazines, pyrrole-type antibiotics, pyro-compounds, and indole derivatives. A small number of antibiotic like compounds that do not contain nitrogen have also been isolated from fluorescent pseudomonads; among these compounds is 2, 4-diacetylphloroglucinol (DAPG), which has been used in the control of plant root diseases [24]. Important antimicrobial metabolites include the phenazines, such as phenazine-l-carboxylate (PCA) [25] and the c-acetyl-phloroglucinols [26], which are effective against 'take-all' of wheat. Both of these compounds have been isolated from microcosm rhizospheres colonized by the producing strain, but not from roots colonized by mutant's defective in the metabolite synthesis pathway [27]. Phloroglucinol and pyoluteorin have been shown to be largely responsible for the prevention of' damping off of sugar-beet (caused by *Pythium ultimum)* and cotton (caused by *Pythium* spp.), whereas phloroglucinol and hydrogen cyanide (HCN) are responsible for the control of black root-rot of tobacco (caused by *Thielalfiwsis basicola)* by *P. fluorescens* CHA0.

2, 4- DIACETYLPHLOROGLUCINOL (DAPG) A POTENTIAL BIOCONTROL METABOLITES:

2, 4-DAPG is a phenolic compound with broad spectrum antifungal, antibacterial, anthelminthic, and phytotoxic activity [28]. Numerous studies have demonstrated the determinative role of DAPG production in the suppression of a variety of soilborne diseases by fluorescent pseudomonads. It exhibits biocontrol activity against phytopathogens, such as the oomycetes, *Pythium ultimum* and *Phytophthora cactorum* and the fungus *Fusarium oxysporum and Thielabiopsis basicola* affecting several plant crops, including sugar-beet [29], tomato, tobacco and strawberry and is antagonistic toward the potato-cyst nematode *Globodera rostochiensis.*

Evidence for the function of DAPG in disease suppression has chiefly come from studies on DAPG-deficient mutants which gave reduced plant protection and from the demonstration of DAPG production in the rhizosphere [30]. DAPG-producing pseudomonads are commonly found in the rhizosphere of important crops such as cucumber, maize, pea, tobacco, tomato, and wheat, and they have been shown to be an important biological factor of the natural suppressive of certain agricultural soils to take all disease of wheat and black root of tobacco.

Confirmation for importance of 2, 4-DAPG in plant protection also comes from studies on 2, 4-DAPG-negative mutants of *Pseudomonas fluorescens* and nonproducing strains into which 2, 4-DAPG biosynthetic plasmids have been transferred. By this approach,

it has been verified that 2, 4-DAPG contributes to the control of black root rot of tobacco, take-all of wheat, *Pythium* damping-off of sugar beet, bacterial soft rot of potato and potato cyst nematodes. In situ detection of 2,4-DAPG in the rhizosphere, i.e., at the site of disease suppression, of plants treated with producing strains further supports the role of the metabolite in plant protection [31].

ORGANIZATION AND REGULATION OF THE DAPG BIOSYNTHETIC OPERON:

In strains *P. fluorescens* Q2-87 and CHAO, the four biosynthetic genes, phlACBD are organized as an operon (Fig. 2) and are indispensable for the production of DAPG as well as monoacetylphloroglucinol (MAPG), which is a precursor or degradation product of DAPG. The biosynthetic operon is flanked by the divergently transcribed phlF gene encoding a repressor protein of the DAPG synthesis and a gene coding for a putative efflux protein (phlE) (Fig. 2) [33,34,35]. In addition, Schnider-Keel et al. [34] have sequenced two new open reading frames downstream of phlF, designated phlG and phlH (Fig. 2).

At a transcriptional level, DAPG biosynthesis is regulated by the pathways specific repressor phlF in *P. fluorescens* Q2-87, Fl 13, and CHAO. PhlF exhibits a helixturn- helix motif typical for DNA binding proteins and has been shown to bind to the phlA, phlF intergenic region. Overproduction of PhlF resulted in repression and inactivation of phlF in depression of DAPG biosynthesis [34]. Repression mediated by PhlF can be influenced by a range of secondary metabolites produced by rhizosphere microorganisms. Schnider-Keel et al. [34] have shown a positive autoregulation of DAPG in CHAO mediated through PhlF. In addition, the microorganism's extracellular metabolites pyoluteorin and salicylate as well as fusaric acid (FA), a pathogenicity factor of Fusarium oxysporum, exert a negative impact on DAPG production.

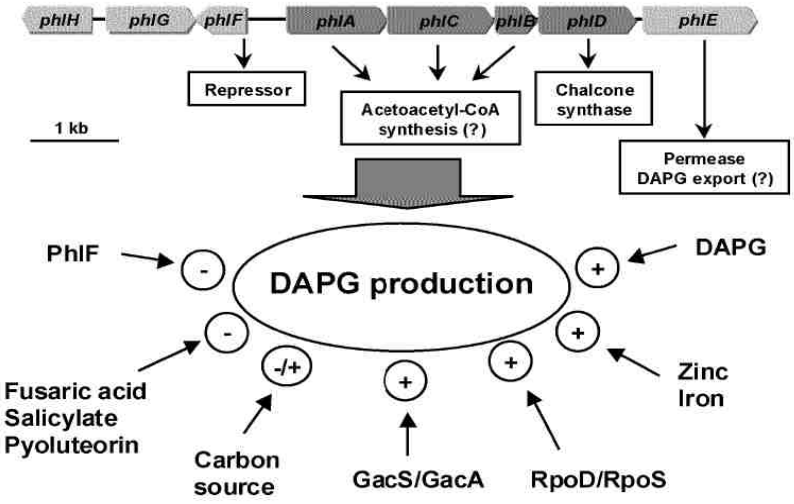

Fig 2: Genetic organization of DAPG genes [34,35]

The negative regulation mediated by salicylate and FA requires a functional PhlF protein. Additional control of DAPG biosynthesis genes at the transcriptional level is proposed for the housekeeping sigma factor σ^D (rpoD) and the stationary phase factor σ^S (rpoS). Amplification of the rpoD gene encoding σ^D in *P. fluorescens* CHAO resulted in an enhanced DAPG production and protection of cucumber against *Pythium ultimum* was improved. Conversely, an RpoS$^-$ mutant overproduced DAPG in strain Pf-5 resulting in enhanced biocontrol of cucumber against *P. ultimum* [36]. Although a DAPG-regulatory function for the heat shock factor (σ^H) has never been demonstrated, its negative impact on pyoluteorin production in Pf-5 has been shown by Whistler et al [37]. The two-component system gacS/gacA globally regulates production of extracellular enzymes and secondary metabolites including DAPG [38]. Like in other prokaryotic two-component systems, the membrane bound sensor kinase (GacS) functions as an environmental sensor and activates the cognate response regulator (GacA) by phosphorylation (fig. 3) The response regulator undergoes a conformational change, which is thought to lead to transcriptional regulation of target genes [39]. Mutations in either gene blocks biosynthesis of extracellular enzymes, DAPG, and other secondary metabolites [40]. Biocontrol of *Thielaviopsis basicola* induced black root rot of tobacco is drastically reduced in a gacA mutant of *P. fluorescens* CHAO. In this strain however, gacA is required for suppression of soil borne diseases of dicotyledons only and not of Gramineae [41].

It has been demonstrated that GacA dependence of cyanide synthase genes (hcnABC) and the protease gene (aprA) in CHAO operates via a post transcriptional mechanism involving the repressor protein RsmA (Fig. 3) [42]. RsmA is suggested to bind to a distinct recognition site overlapping the ribosomal binding site (RBS) of the target RNA and thus blocks its translation (Fig. 3) [42]. In *P. fluorescens* CHAO, RsmA overexpression mimicked partial loss of GacA function and mutational inactivation of rsmA partially suppressed a gacS defect. Moreover, GacA positively regulates a regulatory RNA, called RsmZ [43]. Overexpression of rsmZ suppressed the negative effect of gacS and gacA mutations and inactivation of rsmZ resulted in reduced expression of GacS/GacA target genes. According to the model, RsmZ is thought to sequester the repressor protein thus allowing the translation of the respective mRNA's.

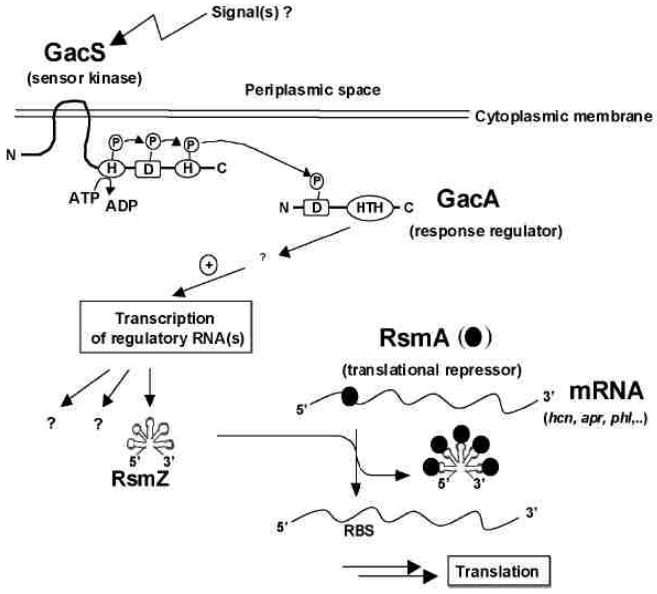

Fig 3: Post transcriptional regulation mechanism involving the two component system involving GacS and GacA in *P. fluorescens* CHAO [35] [44]

PHYSIOCHEMICAL FACTORS INFLUENCING 2, 4-DAPG PRODUCTION:

When strain F113 was grown in volumes of SA broth such that the liquid depth was less than 1 cm, the amount of antibiotic produced was greatly increased (>500%) [24]. Similar quantities of DAPG were also recorded from strain F113 grown on semisolid agar. Monitoring antibiotic production by strain F113 on both semisolid SA agar and in small volumes of SA broth showed that maximum antibiotic production occurred between 4 and 8 days, with negligible increases thereafter. Lower percentages of oxygen supplied to the growing cells resulted in a significant decrease in the number of F113 cells present and thus in the amount of antibiotic detected. However, when DAPG concentration was correlated with the number of CFU in the cell suspension, no significant variation was observed with different oxygen levels supplied in the gas phase. Similarly, when resting cell suspensions pregrown at normal aerobic conditions were exposed to various oxygen levels for 8 days, no apparent differences in DAPG production were observed. However, when no oxygen was added to the gas phase, a significant reduction in DAPG production occurred.

The ratio of the culture volume to the total surface area in a growth container affects the production of DAPG of strain F113[24]. This was investigated by adding increasing amounts of agar to a broth culture. When the agar concentration was increased from 0 to 1.5%, the amount of antibiotic produced per 10^5 CFU increased from 0.46 to 5.21 ppb. However, further increases in agar concentration did not significantly affect DAPG concentration. In addition, increasing the available surface area by sequentially adding

sterilized 1- to 2-mm granite chips to a broth culture resulted in a corresponding increase in DAPG production. This effect did not increase indefinitely, suggesting that there is a minimum amount of surface contact required for optimum DAPG production.

Negligible DAPG production occurred at 37°C, even though all cells remained viable and maximum growth was observed at 12 °C. Other parameters in soil that are known to affect microbial metabolite production are pH and iron concentration. Adding iron concentrations in the range 0 to 200 µM FeCl3 did not affect DAPG production by pregrown cells of strain F113 under the assay conditions used. Similarly, when pregrown cells were resuspended in assay solutions ranging in pH from 2 to 10, there was no change in DAPG produced per viable CFU. Different carbon sources influenced the production of DAPG by strain F113. Cells incubated in the presence of sugars and amino acids, fructose, sucrose, and mannitol promoted high yields of DAPG, whereas no DAPG production was observed when cells were incubated in glucose and asparagine, even though all cells remained viable. This indicates that the type of carbon source greatly influences the production of DAPG by strain F113.

CONCLUSION:

The evidence that microbial metabolites produced by *Pseudomonas fluorescens* specially 2, 4- Diacetylphloroglucinol plays a key role in the biological control of certain key plant pathogens provides a strong incentive to develop such material as biocontrol agents for commercial use. The use of microbial strains producing antimicrobial metabolites in controlled environments, such as horticulture in greenhouses, offers the best possibility for success in the short term. The major challenges to be resolved prior to widespread commercial exploitation of biocontrol strains lies in the ability to predict more confidently the behavior of such strains in the field. Fortunately, as interest in these organisms grows, more information on the genetics, physiology and ecology of metabolite production is becoming available. Such data are of immense importance for the selection of wild-type strains with desirable traits from nature and to provide a more rational framework for the choice of strains for use in inoculants consortia.

Recombinant DNA methods have enabled genetic manipulation of metabolite production with promising results. In this context, research is also directed towards the evaluation of possible risks associated with the large-scale release of these genetically modified organisms (GMOs). Based on the knowledge that is now being accumulated, particularly in the area of molecular microbial ecology of the rhizosphere and metabolite

regulation, improved or manipulated biocontrol agents should become more predictable and reliable for use under field conditions.

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